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A Novel Monoclonal Antibody With a Mono-specificity for a 46 ku-Cytokeratin

FENG Jing[1,2,3], SHEN Yi[2)], YANG Dong-Ling[2)], YAN Xi-Yun[2)*]

([1)]*State Key Laboratory of Microbial Resources, Institute of Microbiology, The Chinese Academy of Sciences, Beijing* 100080, China;
[2)]*National Laboratory of Biomacromolecules, Institute of Biophysics, The Chinese Academy of Sciences, Beijing* 100101, China;
[3)]*Graduate School of The Chinese Academy of Sciences, Beijing* 100039, China)

Abstract A mAb T2-2 was generated using hybridoma techniques, and its target was identified as a 46 ku-cytokeratin (CK), based on biochemical study and a completely overlapped binding pattern of mAb T2-2 with anti-pan-CKs antibodies. An epithelia-specificity of the mAb T2-2 was determined by screening 68 human normal and 65 tumor tissues using immunohistochemistry. Unlike most of anti-CKs antibodies, the mAb T2-2 recognized a mono-specific epitope only expressed on the 46 ku CK, suggesting that mAb T2-2 is superior to most anti-CKs antibodies that cross-reacted with many different kinds of CKs. In addition, it was found that the mAb T2-2 was multipurpose with a broad applicability to ELISA, immunohistochemistry, immunofluorescence, Western blotting, and was also compatible with various fixation reagents. These results strongly indicate that the mAb T2-2 has potential applications for studying CKs function and for diagnosis of tumor and other disorders.

Key words monoclonal antibody, cytokeratins, mono-specificity

Intermediate filaments (IFs), a superfamily of fibers with a diameter of 10 nm, is one of the three components of eukaryotic cytoskeleton, which is distinguished from the other two, microtubules (23 nm) and microfilaments (6 nm)[1]. In contrast to the conservation of microtubules and microfilaments, the expression of IFs is remarkably cell-specific. IFs have been grouped into five types, in which type Ⅰ and type Ⅱ, known as cytokeratins (CKs)[1,2], are the largest and complex constituents of IFs in epithelial cells, consisting of acidic CKs (type Ⅰ, CK9～20) and neutral-basic CKs (type Ⅱ, CK1～8)[1～3]. All CK members have a conserved α-helical rod domain, flanked by diverse N-terminus and C-terminus domain[4,5]. As a rule, CKs assemble as obligatory heterodimer at 1∶1 ratio of any combination of one acidic CK and one neutral-basic CK[1]. For example, CK8/CK18 is a specific pair composing the IFs in simple epithelial cells[6], whereas a CK5/CK14 pair is expressed in basal cell layer of stratified epithelia[7].

It is well-known that CKs, along with the other constituents in IFs, play a critical role in cell structural integrity[8], division, intercellular junction and communication. Recently, increasing studies have demonstrated that CKs also functioned in cell signal transduction[9,10], migration[11], transformation[12], apoptosis[13,14], and drug resistance[15]. In addition, CKs have been found to be the substrate of caspase cleavage in the epithelial cells apoptosis[16]. The soluble fragments of CKs in serum released from proliferating or apoptotic epithelial cells provided useful markers for cell malignancy[17]. For instance, serum fragments of CK8, CK18 and CK19, named as tissue polypeptide antigen (TPA), one major epitope of CK18, namely tissue polypeptide specific antigen (TPS) and CK19 fragment, referred to as CYFRA 21-1, have been widely used in early diagnosis and prognosis of many carcinomas originally derived from epithelial cells[18,19]. Therefore, it becomes important to find and evaluate the significance of CKs used as novel tumor markers[20,21].

The monoclonal antibody (mAb) to CKs was a useful tool for studying the basic mechanisms of the cell progress CKs involved and the application of CKs

*Corresponding author .
Tel: 86-10-64888584, E-mail: yanxy@sun5.ibp.ac.cn
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in tumor diagnosis. Due to the conserved central domain shared by different CKs, many anti-CKs mAbs have a cross reactivity with several different CK polypeptides, even to other types of IFs[22]. Thus, mAbs with mono-specific epitope to a single CK are in demand.

In this present study, we aimed to raise a mAb against CKs. A colon carcinoma cell line LS 174T that expresses CKs was used as an antigen to generate mAb by hybridoma technology. A mAb T2-2 was selected using ELISA and immunohistochemistry. The T2-2 target has been identified as a CKs-correlated protein expressed in LS 174T and colon carcinoma tissues, hepatoma and many other carcinoma tissues, as well as their corresponding normal tissues. Our data provide a useful tool for studying the biological function of CKs and for application in diagnosis of tumor and other disorders.

1 Materials and methods

1.1 Cell lines, tissues and mice

Human hepatocellular carcinoma cell lines SMMC 7721, HepG2, human colorectal adenocarcinoma cell line LS 174T and other cell lines used in this study were obtained from American Type Culture Collection (Manassas, VA, US) and kept in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, US) supplemented with 10% fetal bovine serum (FBS) at 37℃ in humidified CO_2 incubator. Human tumor tissues were obtained from the tissue bank of the 301 Hospital in Beijing. Human normal tissues were obtained from the Beijing Legal Medical Institute. BALB/c nude mice were purchased from the Animal Center of the Chinese Academy of Medical Science, Beijing.

1.2 Generation of monoclonal antibody

BALB/c mice were immunized by intraperitoneal injection with 1×10^7 LS 174T cells lysate combined with Freund complete adjuvant, and boosted 4 times weekly, and then their spleens were taken for hybridoma preparation as described[23].

MAbs were obtained from hybridoma culture supernatant and ascites. For ascites preparation, female BALB/c mice (6～8 weeks old) were first injected intraperitoneally with 0.5 ml Pristane, and 10 days later 5×10^6 hybridoma cells were inoculated to their peritoneal cavity. Ascites was taken from the mice 2 weeks later.

1.3 Screening of antibodies with ELISA and immunohistochemistry

ELISA was used to select antibodies binding to LS 174T cells. Cells were grown in 96-well plate up to sub-confluence, washed with PBS, and then fixed with chilled methanol/acetone (1 ∶ 1) for 1 min. After 3 times washing with PBS, plates were incubated with hybridoma culture supernatants at room temperature for 1 h, and the bound antibodies were detected by incubation with HRP-conjugated anti-mouse IgG (Sigma, Deisenhofen, Germany) for another 1 h. After careful washing, 3,3′,5,5′-tetramethylbenzydine(TMB) was added as substrate. The color reaction was measured at 450 nm using a BIO-RAD microplate reader (BIO-RAD, Hercules, CA, US).

For immunohistochemistry, cryosections from human normal or tumor tissues were fixed with chilled acetone, and then incubated with 3% H_2O_2 in methanol to quench endogeneous peroxidase. The sections were blocked with 5% horse serum in PBS, and incubated with mAb ascites diluted at 1 ∶ 1 000 with PBS in a moist chamber overnight at 4℃, followed by incubation with biotinylated anti-mouse IgG, (Vector Laboratories, Burlingame, CA, US) for 1 h at 37℃ and finally with HRP-conjugated streptavidin (Vector Laboratories) at 37℃ for 1 h. After every incubation step the sections were washed 3 times with PBS. Next, the sections were reacted with substrate DAB and counterstained with hematoxylin, followed by observation under a microscope. For negative controls, the primary antibodies were omitted.

1.4 Indirect immunofluorescence microscope

Cell monolayers grown on coverslips were fixed 1 min with either chilled methanol/acetone (1 ∶ 1) or 4% formaldehyde. The coverslips were blocked with goat serum diluted with PBS for 30 min at room temperature, incubated with mAb T2-2 ascites diluted at a 1 ∶ 1 000 in PBS for 1 h at 37℃ and subsequently with goat anti-mouse IgG-FITC antibody (Sigma) for 40 min at 37℃.

For double staining, the coverslips were incubated with both mAb T2-2 and rabbit anti-pan-CK polyclonal antibody (Santa Cruz, CA, USA). The secondary antibodies used were goat anti-mouse IgG-FITC and anti-rabbit IgG-Cy3 (Sigma). Specimens were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) at 10 mg/L for 30 min at room temperature. After every incubation step the sections

were washed 3 times with PBS. Finally, the coverslips were examined using fluorescence microscope or confocal laser scanning microscope (Olympus, Japan).

1.5 Western blot assay

Human tumor tissues from surgical resection were homogenized with a tissue grinder at 4℃ in TNE lysis buffer containing 50 mmol/L Tris·HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L PMSF and complete protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA) solution. Alternatively, monolayer cells grown in dish were lysed with 1 ml TNE lysis buffer for 30 min at 4℃. The lysates were pelleted by centrifugation at 12 000 r/min for 10 min at 4℃, and the pellets of cells were dissolved with TNE buffer containing 8 mol/L urea. The supernatants and pellet solutions were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% non-fat milk in PBS for 2 h at room temperature, the membranes were probed either directly with HRP-conjugated anti-mouse IgG (Pierce, Rockford, IL, USA) or with mAb T2-2 followed by HPR-conjugated anti-mouse IgG (Pierce). Protein bands were visualized with luminescence substrate (Pierce).

2 Results

2.1 Generation and selection of mAb T2-2

We used a whole cell lysate of LS 174T as antigen to raise mouse monoclonal antibodies by hybridoma technology. ELISA was applied for screening positive clones to LS 174T cells. Over 300 clones of hybridoma, a monoclonal antibody termed as T2-2 was selected. The mAb T2-2 was identified as IgG1/κ and showed specific, constant and intensive binding activity to the target cells.

2.2 Specificity of mAb T2-2 on human tissues and cell lines

To investigate the specificity of mAb T2-2 to human tissues, we screened 68 human normal and 65 tumor tissue cryosections using immunohistochemistry. The results were summarized in Table 1. The mAb T2-2 staining was found in a broad spectrum of normal and tumor tissues. Hepatoma, colon cancer and rectal cancer showed the more intensive binding signal especially. However, no significant binding of the mAb T2-2 was observed in normal brain, heart, spleen or tumor tissues from esophagus cancer.

Table 1 Immunohistochemistry assay of mAb T2-2 specificity to human tissues

Normal tissue	Positive/Cases	Tumor tissue	Positive/Cases
Lung	3/3	Lung cancer	10/10
		Esophagus cancer	0/2
Stomach	3/3	Gastric cancer	5/5
Small intestine	4/4		
Large intestine	1/1		
Colon	3/3	Colon cancer	11/11
		Rectal cancer	14/14
Liver	2/3	Hepatoma	14/14
Gallbladder	0/1		
Pancreas	3/3		
		Breast cancer	2/2
Ovary	1/4	Ovary cancer	1/3
Oviduct	3/3		
Endometrium	2/2	Endometrial carcinoma	1/1
Cervix	2/2	Cervical carcinoma	2/2
Kidney	4/4		
Bladder	0/3		
Prostate	2/2		
Testicle	1/4		
Spleen	0/4		
Thyroid	2/2		
Adrenal	0/2		
Heart	0/2		
Brain	0/2		
Skin	2/4		
Follicle	4/4		
Naevus	3/3	Melanoma	1/1

The typical binding patterns of the mAb T2-2 in normal and tumor tissues were represented in Figure 1, which showed an epithelia-specific staining of the mAb T2-2 in these tissues. Figure 1f showed that the mAb T2-2 stained the cytoplasm of colon gland epithelia assembled in a garland form. The T2-2 epithelia-specific binding patterns were also found in other tissues (data not shown). Figure 1g and Figure 1h showed the binding of T2-2 to hepatoma and colon carcinoma, respectively. The malignant hepatocyte and the branched colon gland were stained intensively by mAb T2-2.

To further test the specificity of the mAb T2-2 on cell level, we used indirect immunofluorescence assay to investigate the T2-2 binding pattern on human cell lines. As listed in Table 2, 14 out of 20 cell lines tested were T2-2-positive, including four colorectal adenocarcinoma cell lines and three hepatocellular carcinoma (HCC) cell lines. No obvious binding of

T2-2 was observed on lung carcinoma cell A549, melanoma cell A375 or mammary gland adenocarcinoma cell MCF-7. The two glioma cell lines tested, BT-325 and U251, were also not detected by mAb T2-2.

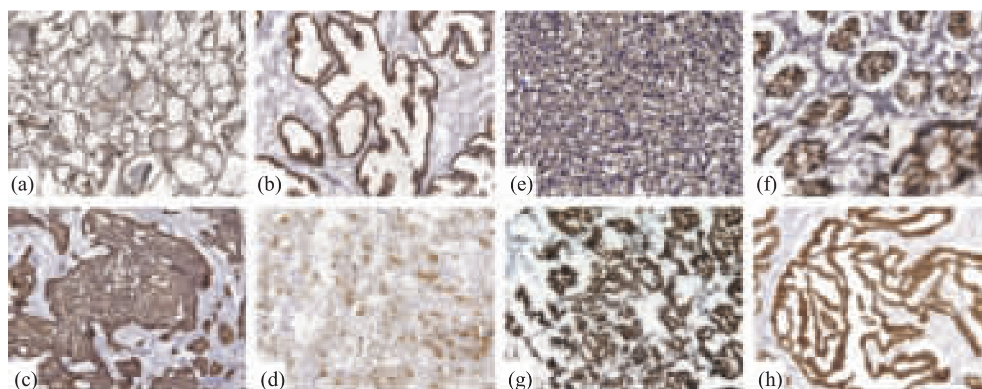

Fig. 1 Immunohistochemistry analysis of T2-2 antigen on frozen sections from human normal or tumor tissues
The mAb T2-2 recognized epithelia-specific antigen in the epithelial cells, including the epithelia of thyroid follicle (a), prostate gland (b), cervix (c), stomach (d), hepatocyte (e) and glandular epithelia of colon (f. The bottom right area showed a magnification of a colon gland, which revealed a strong T2-2 staining in the cytoplasm of the simple columnar epithelia). Tissues from hepatoma (g) and colon carcinoma (h) showed the similar binding pattern of T2-2 with (e) and (f), respectively. The original magnification of (e), (f), (g) was 200×; the other, 100×.

Table 2 Indirect immunofluorescence assay of mAb T2-2 immunoreactivity to human tumor cell lines

Cell line	Immunoreactivity for T2-2
Bladder carcinoma 5637	+
Bladder carcinoma T24	+
Choriocarcinoma BeWo	+
Colorectal adenocarcinoma HT-29	+
Colorectal adenocarcinoma LS 174T	+
Colorectal adenocarcinoma SW1116	+
Colorectal adenocarcinoma SW948	+
Duodenum carcinoma HuTu 80	−
Glioma BT-325	−
Glioma U251	−
Hepatoma Alex	+
Hepatoma HepG2	+
Hepatoma SMMC7721	+
Lung carcinoma A549	−
Mammary gland adenocarcinoma MCF-7	−
Mammary gland ductal carcinoma ZR-75-1	+
Melanoma A-375	−
Ovary adenocarcinoma SKOV-3	+
Pancreas carcinoma Capan-2	+
Pancreas carcinoma SW1990	+

In immunofluorescence assay, we found an interesting observation. The mAb T2-2 recognized its antigen only on the pre-fixed HepG2 cells either with methanol/acetone (Figure 2a) or with 4% formaldehyde (data not shown), but did not bind to the non-fixed HepG2 cells (Figure 2b). In control groups, FITC-conjugated secondary antibody did not react with any HepG2 cells either pre-fixed or non-fixed

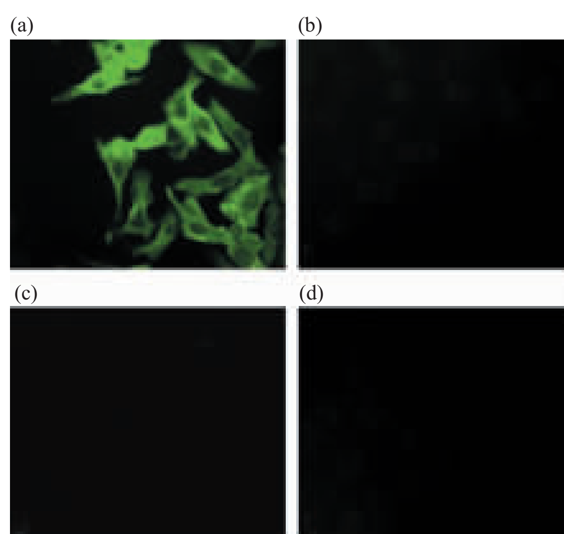

Fig. 2 Indirect immunofluorescence imaged the binding of T2-2 to HepG2 cells
MAb T2-2 showed an intensive binding to HepG2 which had been pre-fixed with methanol/acetone (a), but did not stain the non-fixed HepG2 (b). The FITC-conjugated secondary antibody did not show any reaction with HepG2 cells either pre-fixed (c) or non-fixed (d). The original magnification was 200×.

(Figure 2c and 2d), excluding the cross-reactivity caused by secondary antibody.

The fixation-dependent binding activity, together with the network binding pattern in the cytoplasm, clue in the information that the T2-2 antigen seems more likely a cytoplasm-localized protein related to cytoskeleton.

2.3 Biochemical characterization of T2-2 antigen

To investigate the T2-2 target molecule, we performed SDS-PAGE and Western blot assay to analyze the specimens from LS 174T cells, colon carcinoma and hepatoma tissue. As shown in Figure 3, the mAb T2-2 specifically recognized a unique protein band with a molecular mass of 46 ku. We also found that T2-2 antigen appeared same size in both tumor tissues and LS 174T cell lines. Whereas anti-pan-CKs polyclonal antibody used as a positive control recognized a series of protein bands in the LS 174T lysate.

One remarkable biochemical property of T2-2 antigen was found that the antigen was insoluble and undetectable in the TNE-lysis buffer (Figure 3, lane *3*). When 8 mol/L urea was introduced into the lysis buffer, the T2-2 antigen became soluble and detectable as determined by immunoblot assay (Figure 3, lane *4*). This character provides another evidence that the T2-2 antigen is a CK protein.

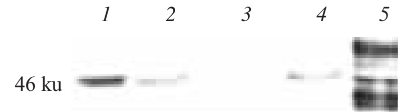

Fig. 3 T2-2 target was identified by immunoblots assay
T2-2 recognized a protein (46 ku) in the lysate of colon carcinoma (lane *1*) and hepatoma (lane *2*). For LS 174T lysate, T2-2-recognized antigen was not detectable in the supernatant (lane *3*) but it was detected in the urea-dissolved pellet (lane *4*). As a positive control, the rabbit anti-pan-cytokeratin polyclonal antibody recognized a series of CK proteins in the urea-dissolved pellet (lane *5*).

2.4 Identification of T2-2 antigen

To test our hypothesis that T2-2 antigen may be one of cytokeritin proteins, we double-stained three cell lines, HepG2, SMMC 7721 and LS 174T, with mAb T2-2 and anti-pan-CKs rabbit polyclonal antibody, followed by FITC-labeled anti-mouse IgG and Cy3-labeled anti-rabbit IgG. Confocal microscope images were shown in Figure 4. T2-2 antigen was located in the cytoplasm, with interlaced filament-like structure stretching from the nucleus to the surface membrane in all the three cell lines. The mAb T2-2-bound network structure of LS 174T was more compact than that of HepG2 and SMMC 7721. The staining pattern of mAb T2-2 looked very similar to that stained by the anti-pan-CKs antibodies. In the double staining assay, the T2-2-staining pattern was

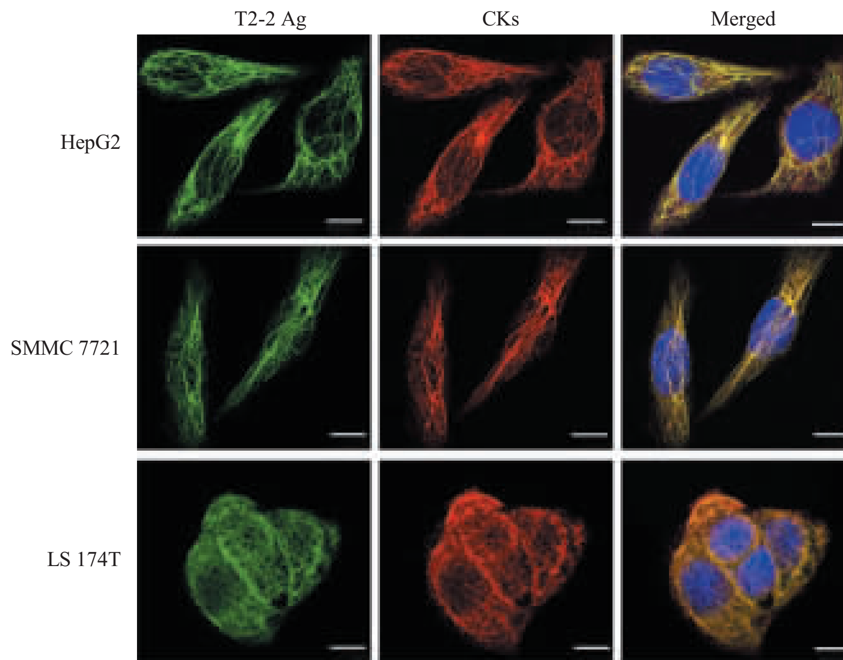

Fig. 4 Indirect immunofluorescence assay showed that T2-2 antigen was co-localized with CKs
Three human carcinoma cell lines, HepG2, SMMC 7721 and LS 174T were labeled intensely by either mAb T2-2 (green) or anti-CKs polyclonal antibodies (red). The double staining of mAb T2-2 and anti-CKs showed merged images, in which nuclei were stained with PI (blue). Bars, 10 μm.

completely overlaid by anti-pan-CKs polyclonal antibody in the three different cell lines. These merged images of T2-2 antigen with CKs suggest that the T2-2 antigen is a CK protein or a CK-related protein. It is clear that mAb T2-2 recognized a mono-specific epitope on this CK protein, which did not cross-react with other CKs.

3 Discussion

In this study, we generated and characterized a mAb T2-2 and identified its antigen as a CK with a size of 46 ku. Unlike most of other anti-CK antibodies against an epitope shared by many different CK proteins, the mAb T2-2 recognized a mono-specific epitope only expressed on this CK, which did not cross-react with other CK proteins. The mono-specific epitope of mAb T2-2 indicates its utility for further study of the CK function and for application in diagnosis of tumor and other disorders.

Although we have not confirmed what kind of CK recognized by the mAb T2-2, our data strongly support that the T2-2 antigen is one of CKs: (1) The epithelia specificity is a remarkable character of CKs. In the immunochemical assay, we found that the mAb T2-2 specifically recognized the epithelia originated tissues, including gland epithelia and hepatocytes. It is noteworthy that the mAb T2-2 did not recognized two non-epithelia-derived cell lines, BT-325 and U251, which have been demonstrated that T2-2 antigen was not the gliacyte-specific IFs, glial filaments. (2) T2-2 antigen was observed in cytoplasm with a cytoskeleton-typical network pattern as determined by immunofluoresence. (3) A characteristic features of IFs proteins that distinguished from other cytoskeletal proteins, is their insolubility. Among IFs proteins, CKs were the least soluble [24]. Our findings that the T2-2 target was only visualized in a fixation-dependent manner, together with its insolubility in aqueous buffer, suggest T2-2 antigen is a CK protein in cytoplasm. (4) The completely overlapped binding of mAb T2-2 with the polyclonal antibodies against pan-CKs provided the most direct evidence to support that the mAb T2-2 was CKs-specific antibody.

The mono-epitope specificity of the mAb T2-2 suggest it was superior to most other anti-CKs-specific antibodies that always cross-reacted with many different kinds of CKs. In addition, we found that the mAb T2-2 was a multipurpose antibody with a broad applicability to ELISA, immunohistochemistry, immunofluorescence and Western blotting. In fact, only 10% existed anti-CKs mAbs were applicable for all these four assays [22]. Moreover, mAb T2-2 was found compatible with various fixative reagents, such as acetone, methanol and formaldehyde.

Compared with the recombinant CKs or the purified CKs commonly used as antigen in generation of mAb against CKs, here we used cell lysate as antigen to generate mAb T2-2 which have several advantages. First, it does not need to express and purify antigen proteins. Second, the antigens tend to keep the natural conformation. Third, it is a practical way to identify novel target based on antibody screening approach. Thus, the utility of native antigen would be an advantaged option for the development of mAbs used as probe tools.

Taken all together, the mAb T2-2 will be a very useful tool for studying CK function and for applying in diagnosis of tumor and other disorders. It will be very interesting to further identify T2-2 antigen and to understand its biological function, and to evaluate its utility in clinical diagnosis.

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一株新型抗 46 ku-细胞角蛋白的单特异性单克隆抗体

冯 静[1,2,3] 沈 毅[2] 杨东玲[2] 阎锡蕴[2]*

([1]中国科学院微生物研究所，微生物资源前期开发国家重点实验室，北京 100080；
[2]中国科学院生物物理研究所，生物大分子国家实验室，北京 100101；
[3]中国科学院研究生院，北京 100039)

摘要 利用杂交瘤技术制备了一株单克隆抗体 T2-2，对其生化性质的研究及其与抗细胞角蛋白多克隆抗体完全重合的定位表明，该抗体特异性识别一种分子质量为 46 ku 的角蛋白. 对 68 例正常组织和 65 例肿瘤组织的免疫组化结果显示，单克隆抗体 T2-2 具有上皮细胞特异性. 与其他多数抗细胞角蛋白抗体常与一种以上细胞角蛋白多肽表现出交叉反应不同的是，该抗体只识别 46 ku 细胞角蛋白多肽上的某一单特异性表位. 另外，单克隆抗体 T2-2 适用于多种免疫实验技术，包括 ELISA、免疫组化、细胞免疫荧光及蛋白质印迹等，而且适用于多种固定剂. 以上结果表明，单克隆抗体 T2-2 将成为细胞角蛋白功能研究和肿瘤诊断的有力工具.

关键词 单克隆抗体，细胞角蛋白，单特异性
学科分类号 Q819

* 通讯联系人. Tel: 010-64888584, E-mail: yanxy@sun5.ibp.ac.cn
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